

Temperature Sensitive Shut-off of Alphavirus Minus Strand RNA Synthesis Maps to a Nonstructural Protein, nsP4

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Minus strand RNA synthesis by the positive strand alphaviruses, Sindbis and Semliki Forest viruses, normally occurs early in infection, is coupled to synthesis of viral nonstructural proteins and to formation of viral replication complexes, and terminates and does not occur late in infection. Previously, ts24 of the A complementation group of Sindbis virus RNA-negative mutants was found to possess, among its other temperature sensitive defects, a temperature sensitivity in the normal cessation of minus strand synthesis which enabled minus strands to be synthesized late in infection at 40° in the absence of protein synthesis. Revertants of ts24 (ts24R1, ts24R2) retained the defect in the shutoff of minus strand synthesis, indicating the lesion was not conditionally lethal and could map outside the A cistron. The studies reported here used an infectious clone of Sindbis virus to identify the mutation responsible for this phenotype. Hybrid viruses were prepared from constructs containing restriction fragments of the cDNA of ts24R1 in place of the corresponding fragments in the infectious SIN HR clone and screened for their ability to synthesize minus strands at 40° in the presence of cycloheximide. A unique base change of an A for a C residue at nt 6339, predicting a change from glutamine to lysine at amino acid 195 in nsP4, was found in genomes of ts24, ts24R1, and ts24R2. Other nucleotide changes present at the 5' and 3' termini did not affect minus strand synthesis. The substitution of the parental Sindbis virus sequence that encompassed nt 6339 in an infectious clone of the ts24R1 revertant eliminated the mutant phenotype. We conclude that the ability to continue minus strand synthesis at 40° exhibited by ts24 and its revertants is caused by an alteration in nsP4, which is the alphavirus replicase or an essential component of the replicase. We hypothesize that this domain of nsP4 functions to fix the minus strand as the stable template of alphavirus replication complexes. © 1990 Academic Press, Inc.

INTRODUCTION

Sindbis virus, SIN, is an enveloped, positive strand RNA virus and a member of the alphavirus genus of the family Togaviridae (reviewed, Strauss and Strauss, 1988). The synthesis of 49 S genome RNA and 26 S subgenomic mRNA are dependent on the synthesis of genome-length minus strand RNA. Normally, alphavirus minus strand RNA synthesis occurs early after infection, requires concurrent protein synthesis, and terminates (Sawicki and Sawicki, 1980). We (Sawicki *et al.*, 1981a) identified a temperature sensitive, RNA-negative mutant of the heat-resistant (HR) strain of SIN, SIN HR ts24, assigned to the A complementation group (Burge and Pfefferkorn, 1966a,b; Strauss *et al.*, 1976), which failed to turn off minus strand synthesis late in infection and lost the sensitivity of minus strand RNA synthesis to inhibition of protein synthesis if the infected cells were shifted to 40°, the nonpermissive temperature, and hypothesized that this mutant contained a temperature sensitive lesion in a viral non-

structural protein that acted to turn off minus strand synthesis. Subsequently, we (Sawicki and Sawicki, 1986a) isolated revertants of ts24, e.g., ts24R1 and ts24R2, that retained the temperature sensitive defect in the cessation of minus strand synthesis but were no longer temperature sensitive for growth or for RNA synthesis, i.e., they were no longer RNA negative mutants. Therefore, temperature sensitivity in the termination of minus strand synthesis was not conditionally lethal and the mutation responsible for this phenotype, which we call the ts24R1 phenotype, could map outside of the A cistron (Sawicki and Sawicki, 1985). Further studies by us (Sawicki and Sawicki, 1986a, 1987) on ts24 and the revertants of ts24 suggested that the ts24R1 phenotype, i.e., the ability to continue or to resume the synthesis of minus strands at 40° in the absence of protein synthesis, could be caused by the replication complexes exchanging templates rather than to a mutation in a viral protein responsible for turning off minus strand synthesis. In this model, newly synthesized plus strands would replace the minus strands which normally form a stable association with the replication complex.

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The mutation in ts24, and in several other A group mutants, responsible for temperature sensitivity of growth and of overall RNA synthesis has been mapped to nsP2 (Hahn *et al.*, 1989b). Because ts24 has several temperature sensitive phenotypes, namely in growth, in synthesis of the 26 S subgenomic mRNA, and in processing of the viral nonstructural polyproteins (Scheele and Pfefferkorn, 1969; Keränen and Kääriäinen, 1979) in addition to the temperature sensitivity of the termination of minus strand synthesis (Sawicki and Sawicki, 1985), we decided to map the mutation affecting the termination of minus strand synthesis using the ts24R1 revertant of ts24 since it retained only the temperature sensitive defect in the shutoff of minus strand synthesis (Sawicki and Sawicki, 1986a). We identified a region of the ts24R1 genome that encoded the ts24R1 phenotype and then identified a unique base change within this region. We report here that a single nucleotide change in the coding region of nsP4 is responsible for the continuation and the resumption of minus strand synthesis at 40° in ts24 and its revertants. These studies were made possible because of the construction of a plasmid carrying a full-length cDNA of SIN from which infectious transcripts can be generated (Rice *et al.*, 1987).

MATERIALS AND METHODS

Virus stocks, growth, and purification

Sindbis virus (SIN) stocks derived either from cDNA clones or from the original SIN heat resistant (HR) parental strain, and temperature-sensitive (ts) mutants and their revertants were grown and titered on monolayers of chicken embryo fibroblasts (CEF). SIN RNA was obtained from purified virions as described previously (Sawicki and Sawicki, 1986a).

cDNA synthesis, purification, and primer extension

The cDNA synthesis and cloning of the genome of ts24R1, a revertant of the mutant ts24 (Sawicki and Sawicki, 1987), was performed using procedures generally outlined previously for the cloning of SIN (Toto1101; Rice *et al.*, 1987). The cloning strategy consisted of obtaining double-stranded cDNA from each of three regions that together covered the entire genome. Unless indicated otherwise, restriction and other enzymes were obtained from New England Biolabs (Beverly, MA).

A cDNA copy of the 5' region was obtained using an oligonucleotide complementary to nt 2806–2823 to prime reverse transcription of the virion RNA and a primer for second strand cDNA synthesis that contained the *Sst*I (BRL, Bethesda, MD) recognition se-

quence followed by the SP6 RNA polymerase promoter sequence (TATGCTAAATCCACTGTGATAT) and the first 15 nucleotides of the 5' terminus of the small plaque strain of SIN HR. Following first strand synthesis the RNA was removed by alkali treatment, the primer for second strand synthesis was annealed to the first strand cDNA, and second strands were synthesized using either *Escherichia coli* DNA polymerase I and *E. coli* ligase or T7 DNA polymerase (Sequenase, USB, Cleveland, OH). The double-stranded cDNA was digested with *Sst*I (whose unique cleavage site is 85 nucleotides upstream of the SP6 promoter sequence in Toto1101) and *Cla*I (whose unique cleavage site is at nt 2713 of the SIN cDNA sequence; Strauss *et al.*, 1984) and ligated to Toto1101, which had been previously cut with the same two enzymes and treated with calf intestinal alkaline phosphatase.

The region from nt 2288 to 7999 of ts24R1 was obtained by priming first-strand synthesis with an oligonucleotide which was complementary to positions 8237 to 8256 and using the enzymatic conditions of Okayama-Berg (1982) for second strand cDNA synthesis. cDNA was digested with *Bgl*II (nt 2288) and *Aat*II (nt 7999), subcloned into Proteus 23, a plasmid whose small size (2.5 kb) enabled easy detection of transformants bearing inserts of the correct size. This region from the ts24R1 genome was substituted for the corresponding region in Toto1101. The 3' third of the ts24R1 genome was also cloned in this fashion except that the oligonucleotide primer used for first-strand synthesis contained the recognition sequence for the *Xho*I restriction enzyme plus dT₁₄. *Ap*I 12-mer linkers were ligated to the cDNA to facilitate cutting by *Xho*I, whose cleavage site would otherwise have been at the extreme end of the DNA, and the cDNA was digested with *Aat*II and *Xho*I (whose unique cleavage site is downstream of the poly(A) sequence at nt 11,718) and cloned into the Proteus 23 plasmid before subcloning into Toto1101 using *Aat*II and *Xho*I.

The 3' recombinants were verified by identifying a unique base substitution at nt 11,643 in the 3' noncoding region of ts24 and its revertants (see Fig. 3) and by their shorter poly(A) sequence. This base change inactivated an *Fnu*4H1 site upstream of the poly(A) in Toto1101 and yielded a 175-nucleotide-long fragment containing the poly(A) sequence when the cDNA was cut with *Xho*I, filled in with the Klenow fragment of *E. coli* DNA polymerase in the presence of radiolabeled triphosphates, then cut with *Fnu*4H1, and the resulting fragments separated on a 8% 7 M urea polyacrylamide gel. The corresponding fragment from Toto1101 was 108 nucleotides long. The 5' recombinants were verified by the absence of a 62-base pair sequence between the *Sst*I site and the SP6 promoter normally

present in Toto1101 (Rice *et al.*, 1987) but deleted from the second strand primer used to prepare the double-stranded cDNA.

Subcloning of smaller fragments of the *Bgl*II to *Aat*II region was accomplished by swapping fragments first into deletion derivatives of π nsP34C, a plasmid which contains the *supF* selective marker and the SIN sequence from a *Pvu*II site at nt 5160 to the *Nco*I site at nt 8038 (Grakoui *et al.*, 1989; kindly provided by Julie Lemm, Washington University, St. Louis). Deletion vectors included one missing 136 base pairs between the *Kpn*I to *Pst*I sites at nt 5824 to 5960 (called π 1.8) and another with a 153-base pair deletion between the *Eco*RV and *Dra*I sites at nt 6878 to 7031 (called π 3.2). Use of these recipient plasmids, carrying internal deletions, for subcloning allowed verification of the specific exchange of the ts24R1 sequence for the original SIN sequence and enabled several restriction sites that were now unique within the subcloned region to be used to facilitate swapping of even smaller fragments. The unique upstream *Spe*I and downstream *Aat*II cleavage sites in the π nsP34C plasmids were used for reinsertion of this entire region into the infectious Toto1101 clone. Using this strategy, we localized the region of the genome encoding the ts24R1 phenotype and sequenced it using six primers that covered this region. The procedure of Chen and Seeburg (1985) was used to denature the double-stranded DNA, which was sequenced by the chain-termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (Tabor and Richardson, 1987; Sequenase, United States Biochemical Corp., Cleveland, OH). The purified virion RNA obtained from SIN HR, ts24, ts24R1, and ts24R2 (a second independently isolated revertant of ts24) was sequenced using the same oligonucleotide primers and AMV reverse transcriptase (Life Sciences Inc., St. Petersburg, FL). The 5' and 3' termini of virion RNAs were sequenced from single-stranded cDNAs using the chemical method of Maxam and Gilbert (1980).

Construction of an infectious clone of ts24R1

An infectious clone of ts24R1 was obtained by ligation together of the clones containing the 5', middle, and 3' regions of the ts24R1 genome. In addition, it was verified that the cloned 5' region contained the identical sequence of the original ts24R1 virus since the specific second-strand primer used to prepare this cDNA contained, at its 3' end, the Toto1101 sequence from nt 1 to 15; ts24R1 differed from the Toto (SIN HR sp) sequence at nt 5 and 14 (presented in Fig. 3). All clones that contained the 5' region of ts24R1 from the *Sst*I site (nt -25) to the *Cla*I site (nt 2713) and that gave rise to infectious RNA had a 5' terminal sequence identical to

the ts24R1 genome (data not shown). Thus, during cDNA synthesis the primer was apparently trimmed, removing both mismatches and allowing faithful copying of the ts24R1 5' sequence. These clones were used directly for the construction of an infectious clone of ts24R1 and for preparation of additional hybrids containing the 5' region.

In vitro transcription and transfection of CEF monolayers

RNA transcripts were synthesized *in vitro* using SP6 RNA polymerase from plasmid DNA digested with *Xho*I restriction endonuclease to produce run-off transcripts. Reaction conditions were as described previously (Rice *et al.*, 1987). To assay directly the infectivity of the transcripts or to produce virus stocks, the transcription mix was used directly for transfection of CEF monolayers treated with DEAE-dextran by the protocol outlined by Rice *et al.* (1987).

RNA labeling

Monolayers of CEF or BHK21 cells in plastic petri dishes, usually 60 mm in diameter, were infected with a m.o.i. of 100. The infection protocol was as described previously (Sawicki and Sawicki, 1985). Actinomycin D (usually at 2 μ g/ml) was included in all media, and was the generous gift of Merck Sharpe & Dohme (Rahway, NJ). Cycloheximide (at 100 μ g/ml) was from Boehringer-Mannheim (Indianapolis, IN). Radiolabeling of RNA was with [5-³H]uridine (38 Ci/mmol; ICN, Irvine, CA) at a final concentration of 200 μ Ci/ml of Dulbecco's modified Eagle's essential medium containing 2 mg/ml BSA and 22 mM HEPES, pH 7.4 (1.5 ml/60-mm petri dish) unless indicated.

Isolation of SIN RF RNA and detection of minus strand RNA synthesis

The procedures used for the isolation of the double-stranded core (RF RNA) of the replicative intermediates and for the determination of minus strand RNA synthesis by hybridization of heat-denatured RF RNA with unlabeled, purified 49 S plus strand RNA were as described (Sawicki *et al.*, 1981a).

RESULTS

Cloning of the ts24R1 genome and mapping of the mutation

The double-stranded cDNA copy of the genome of ts24R1 was obtained in three pieces as described under Materials and Methods. Figure 1 presents the overall scheme that was used to prepare three separate hybrid clones containing either the 5' region, the middle

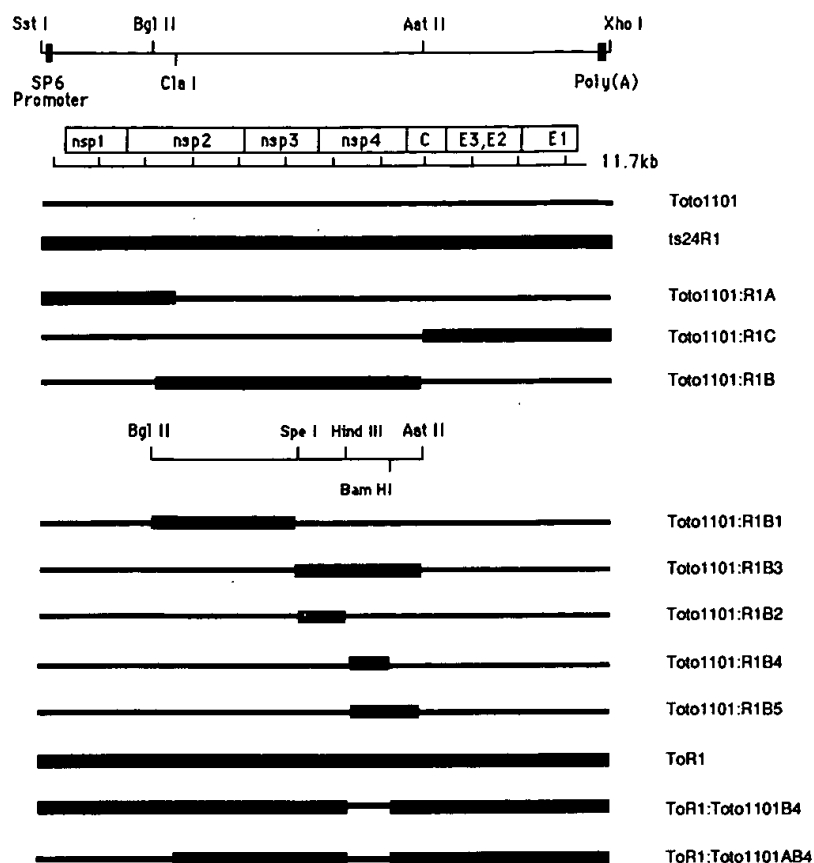


Fig. 1. Construction of hybrid genomes containing different regions of the ts24R1 genome. A schematic of the 11.7-kb genome of SIN cDNA clone Toto1101 (Rice *et al.*, 1987) is shown together with the location of certain restriction sites, the SP6 promoter sequence and the 3' poly(A) sequence. Translated regions are indicated by open boxes for the nonstructural proteins (nsP1-4) and the structural proteins (capsid and the three envelope proteins). The thick dark lines indicate the DNA fragments in Toto1101 that were replaced by ts24R1 cDNA fragments. The thin line indicates the Toto1101 sequence. The names used to refer to the hybrid clones are listed to the right of the figure.

region, or the 3' region of the ts24R1 genome in place of the corresponding region in Toto1101. RNA transcripts of each hybrid clone were prepared using SP6 RNA polymerase and were used to transfect CEF monolayers. Like Toto1101 transcripts, these transcripts contain a single extra G residue at the 5' terminus. The hybrid virus stocks were prepared from cultures maintained at 30° and were assayed for their ability to grow at both permissive (30°) and nonpermissive (40°) temperature. None of the hybrid viruses were temperature sensitive for growth by plaque assay (data not shown), and they synthesized viral RNA to the same extent and at similar rates as SIN HR and ts24R1 (Fig. 2). Thus, dramatic incompatibilities did not result from the presence within the same genomic RNA of both the ts24R1 (SIN HR origin) derived sequences and the Toto1101 sequences (Rice *et al.*, 1987) derived from the small plaque (sp) strain of SIN HR (nt 1-2713 and nt 9804-11,703) and the SIN lab strain of S. Schlesinger (nt 2714-9803).

The hybrid viruses were screened for their ability to continue the synthesis of minus strand RNA under conditions, i.e., late in infection and in the presence of cycloheximide, which do not allow for minus strand synthesis by parental SIN HR. Only the hybrid viruses containing the middle region, encompassing nt 2288 to nt 7999 that codes for part of nsP2, all of nsP3 and nsP4, and part of the capsid protein, reproduced the ts24R1 phenotype (Table 1). This 5.7-kb region [*Bgl*II (nt 2288) to *Aat*II (nt 7999)] was cleaved with *Spe*I and the two resulting fragments exchanged with the corresponding fragments in Toto1101. Only the *Spe*I (nt 5262) to *Aat*II (nt 7999) fragment retained the ts24R1 phenotype. This region was subdivided further with *Hind*III to generate the *Spe*I to *Hind*III fragment (nt 5262 to nt 6267) and the *Hind*III to *Aat*II fragment (nt 6267 to nt 7999) or with *Hind*III and *Bam*HI to produce the nt 6267 to nt 7334 fragment. The ts24R1 phenotype was produced by the sequences bordered by nt 6267 to 7334 which were present in both the *Hind*III to *Bam*HI

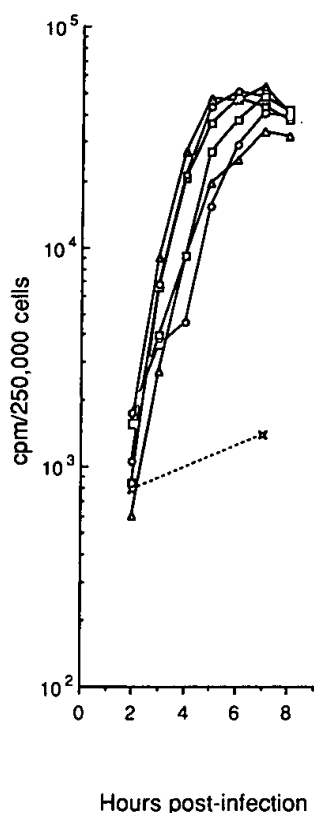


Fig. 2. Kinetics of RNA synthesis by hybrid viruses. CEF cultures were infected with an m.o.i. of 100 and maintained at 30° as described under Materials and Methods. At various times after infection the cultures were pulsed with [³H]uridine for 60-min periods. The acid-insoluble incorporation present at the end of each pulse period is plotted at that time. (□) SIN HR Pfefferkorn; (○) ts24R1; (Δ, Δ, □, ○) viruses from four independent cDNA clones of Toto1101:R1B; (x) mock infected.

and the *Hind*III to *Aat*II fragments and was not produced by those within the *Spe*I to *Hind*III fragment (Table 1). This interval of DNA of approximately 1 kb was sequenced using primers that were specific for this region of the genome. Only one base change, at nt 6339 relative to the Toto1101 and HR sp sequence, was identified in this region (data not shown but see Fig. 4). This base change was present also in the original mutant viruses as determined by sequencing the genomic RNA which was purified from virions of ts24, ts24R1, and ts24R2; it was not present in genomic RNA purified from SIN HR virions. At nt 6339, a C residue in the parental SIN HR sequence [both in Toto1101 DNA and in the genomic RNA purified from SIN HR (Ohio) virions] was changed to an A residue. The change predicted a glutamine (CAG) to lysine (AAG) substitution at amino acid 195 in the nsP4 protein, replacing an uncharged polar residue with a basic amino acid at this position.

Effect of 5' and 3' changes on minus strand synthesis by hybrid viruses

Initially, we had sequenced the ends of the ts24R1 RNA to determine if ts24, ts24R1, and ts24R2 contained a unique alteration in the 3' promoter sequence (Ou *et al.*, 1982; Levis *et al.*, 1986) or in the proposed pan-handle structure that would involve sequences at both the 5' and 3' ends of the 49 S RNA (Strauss and Strauss, 1983) that would be responsible for the ts24R1 phenotype. Any nucleotide changes contributing to the mutant phenotype would be expected to be present in all three mutants showing this phenotype. The changes which were found in both the 5' and 3' noncoding regions of the ts24R1 RNA are shown in Fig. 3. Compared to the Toto1101 (SIN HR sp) sequence, two base changes within the first 15 nucleotides at the 5' end of ts24R1 RNA were found at nt 5 and 14. Two other strains of SIN HR (Ou and Ohio) had the same base at nt 5 (G) as ts24R1. Although the change at nt 14 was unique to ts24R1, it also was not present in ts24 or ts24R2. As shown in Fig. 3, sequence variability was found also at nt 9 (C or T) and nt 35 (A or G) but these changes could not account for the ts24R1 phenotype. These were the only changes found within the first 248 nucleotides of the 5' end, which include the 60 nucleotides of the 5' nontranslated region (Strauss *et al.*, 1984). At the 3' end of the genomes of ts24, ts24R1, and ts24R2, a C in the SIN HR sequence was changed to a T at position 11,643, 60 nucleotides upstream of the start of the poly(A) sequence. Although this change was about forty nucleotides upstream of the proposed 3' promoter sequence, it was unique to the three viruses possessing the ts24R1 phenotype and was not present in six other SIN genomes (Fig. 3). The results reported above indicated that the mutation responsible for the ts24R1 phenotype mapped to nsP4 and not to the 3' region. However, to determine if the unique base change at the 3' end of the RNA enhanced the expression of the ts24R1 phenotype, i.e., increased the magnitude of minus strand synthesis found with Toto1101:R1B and Toto1101:R1B4 hybrid viruses (Table 1) to the level found with the original ts24R1 virus, several other hybrid viruses were constructed and their level of minus strand synthesis compared to that of ts24R1. One construct (Toto1101:R1B4/3') contained the ts24R1 sequences nt 6226 to nt 7334 and nt 11,214 (a *Pvu*II site) to nt 11,718 [the unique *Xho*I site just downstream of the poly(A) sequence] which combined the base change in nsP4 with the base change at nt 11,643. Two further constructs containing the 5' region and the middle region of ts24R1 but containing either the 3' region of the genome (from nt 8000 to 11,743) as the SIN HR sequence

TABLE 2
MINUS STRAND RNA SYNTHESIS BY ts24R1 REVERTANTS

Virus*	Fragment replaced (nt)	Minus strand RNA (cpm/10 ⁴ RF cpm)	Phenotype
Toto1101		220	wt
ToR1	1-11,708	1990	ts
Toto1101:R1B4	6,267-7,334	1390	ts
Toto1101:R1AB4	1-2,713; 6,267-7,334	1840	ts
ToR1:Toto1101B4	6,267-7,334	260	wt
ToR1:Toto1101AB4	1-2,713; 6,267-7,334	430	wt

* The name to the right of the colon indicates the source of the fragment replaced into the infectious clone, which is indicated to the left of the colon. Toto1101 is the infectious clone of SIN HR; ToR1 is the infectious clone of ts24R1. Synthesis of minus strands was determined as described under Materials and Methods and Table 1. The values given are the cpm in minus strands per 10⁴ cpm of total RF RNA obtained from a single experiment.

values of 2465 cpm for the infectious clone of ts24R1 and 60 cpm for Toto1101. In the same experiment, 4720 cpm of minus strand RNA were detected per 10⁴ cpm of RF RNA that was isolated from cells infected with SIN HR and labeled in the absence of cycloheximide from 1 to 4 hr p.i. which is the period encompassing minus strand synthesis (Sawicki *et al.*, 1981b). We conclude differences of twofold or less in minus strand synthesis between hybrid viruses containing the mutant nsP4 and the original ts24R1 were within experimental variation and not significant. Viruses containing the nsP4 mutation without (Toto1101:R1B, B3, B4; Table 1) or with the ts24R1 5' end (Toto1101:R1AB, AB3', Table 1; Toto1101:R1AB4, Table 2) gave values similar to each other or also within a twofold range. Thus, no evidence was found to indicate a role for any sequence other than the nsP4 mutation in this phenotype.

Analysis of ts24R1 revertants

To verify that the base change at nt 6339 in nsP4 was indeed necessary and sufficient for the ts24R1 phenotype, we constructed a revertant of ts24R1 which contained the parental SIN HR base at position 6339. This was accomplished by replacing the *Hind*III to *Bam*HI fragment of the infectious ts24R1 cDNA (ToR1) with the Toto1101 fragment. Sequence analysis had demonstrated that the base change at nt 6339 was the sole difference between these two fragments. Alteration of this single nucleotide in the ts24R1 sequence resulted in the elimination of the mutant phenotype for minus strand synthesis (Table 2). A level of minus strand synthesis equivalent to that of Toto1101

was found for the engineered revertant virus produced from ToR1:Toto1101B4. Furthermore, when both the 5' region from nt 1 to 2713 and the nsP4 mutation were replaced with the Toto1101 sequence in the hybrid virus ToR1:Toto1101AB4, minus strand synthesis was reduced to wild-type levels, further supporting the conclusion that the 5' region of the ts24R1 genome was not necessary for the mutant phenotype. Therefore, we conclude that the base change at nt 6339 in the nsP4 coding region was responsible for the mutant minus strand ts24R1 phenotype of ts24 and its revertants.

DISCUSSION

The unusual phenotype of the SIN mutant ts24 and its revertants (Sawicki *et al.*, 1981a; Sawicki and Sawicki, 1986a) of failing to terminate minus strand synthesis late in infection and of continuing or resuming synthesis of minus strands in the presence of protein synthesis inhibitors at 40° was mapped to a region of the viral genome that encodes nsP4. A single base change at nt 6339 leading to the substitution of lysine for glutamine at amino acid residue 195 of nsP4 was found to be responsible for this phenotype. The nsP4 protein is the alphaviral nonstructural protein implicated in replicase activity by the presence of a sequence, Gly-Asp-Asp, beginning at nt 7158 that is closely related to the sequence Tyr-X-Asp-Asp conserved among RNA-dependent viral replicases (Kamer and Argos, 1984; Haseloff *et al.*, 1984) and by genetic

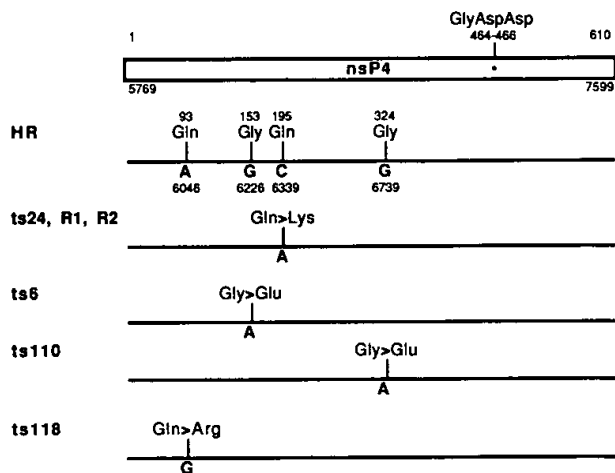


FIG. 4. The location of mutations in nsP4. A schematic of the nsP4 coding region is shown, including its nucleotide (numbered from the 5' end of the genome) and amino acid (numbered from amino acid residue 1 of nsP4) positions (from Strauss *et al.*, 1984) and the location of the conserved Gly-Asp-Asp sequence (Kamer and Argos, 1984). The location of mutations in ts6, ts110, and ts118 are from Hahn *et al.* (1989a). The region sequenced for ts24, ts24R1, and ts24R2 is given in the text.

ts24R1 and ts24R2 does not cause an increase in the rate of plus strand RNA synthesis and in the formation of new replication complexes (Sawicki and Sawicki, 1986a, 1987). We argued that minus strand synthesis at 40° in the absence of protein synthesis in cells infected with ts24R1 was caused by the replacement of the preferred template of the replication complex, i.e., the minus strand, with a plus strand that in turn is copied into a new minus strand (Sawicki and Sawicki, 1986a, 1987). Our original hypothesis (Sawicki *et al.*, 1981a) which stated that ts24 produced a defective, *trans*-active repressor/inhibitor of the viral minus strand polymerase activity has not been supported by data obtained from co-infection experiments. Co-infection of cells with the parental SIN and ts24, which resulted in the synthesis of nonstructural proteins from both viral genomes, did not eliminate minus strand synthesis at 40° in the presence of cycloheximide (Sawicki and Sawicki, 1983). We favor the interpretation that the ts24R1 phenotype results from template switching by old replication complexes rather than from the formation of new replication complexes.

Since the nucleotide change responsible for the ts24R1 phenotype is located in nsP4, the affinity of the nsP4-containing polymerase either directly for promoter sequences which determine template recognition by the replication complex, or indirectly for another polypeptide initiation factor, may have been altered. In addition to minus strands, plus strands would be recognized as templates by the stable replication complex if either newly synthesized, but not yet released 49 S plus strands, or previously released 49 S plus strands replaced minus strands which are normally the preferred templates. Theoretically, therefore, there is evidence for at least two distinct functional regions within the nsP4 protein. The mutation in ts6, and perhaps also in ts110, affects polymerization activities and the mutation in ts24R1 (nt 6339) affects template recognition/binding. It is worth noting that the replicase of the RNA phage Q β which is related to nsP4 also appears to have more than one functional region. Substitution of the glycine residue in the conserved sequence (Tyr-Gly-Asp-Asp) in the Q β replicase led to the loss of enzyme activity *in vivo* but appeared not to affect template recognition activity because the mutant replicase inhibited RNA synthesis by the wild-type replicase (Inokuchi and Hirashima, 1987). Thus, mutation in this conserved domain in the phage protein apparently destroyed template polymerizing activity but not promoter binding. In the case of the alphavirus replicase, the functional form of nsP4 is unknown but it most likely associates with other viral proteins to form a multicomponent replication complex. Therefore, amino acid changes in the nsP4 protein could affect interactions within the com-

plex and thus functions determined by other viral proteins as well as functions that reside specifically in the nsP4 protein. Both the temperature sensitive lesions in ts24R1 and in ts6 can be demonstrated at nonpermissive temperature in replication complexes that were originally synthesized and assembled at permissive temperatures. Thus, these defects are clearly different from those that affect the synthesis, processing, or assembly of the nonstructural polypeptides into functional replication complexes. In alphaviruses the synthesis of minus strands appears to be coupled to the formation of stable replication complexes and to be terminated normally when the synthetic capacity is best put to the production of plus strands. This appears to be determined by a domain of the nsP4 protein which is mutated in ts24, ts24R1, and ts24R2 that functions to ensure the minus strand is the preferred template of the stable replication complex. Whether this region of nsP4 also plays a role in recognizing a promoter site on the plus strand in order to create a replication complex that would synthesize a minus strand remains to be determined.

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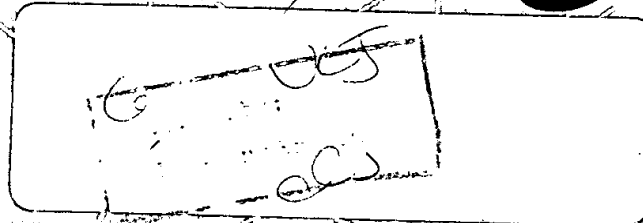
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